Measurement of urinary glycosaminoglycans in dogs

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Objectives—To measure urine concentrations of sulfated glycosaminoglycans (GAGs), determine optimal storage conditions for urine samples, establish a reference range, and determine whether there is correlation between 24-hour total urine GAG excretion and the GAG-to-creatinine ratio (GCR).

Animals—14 healthy adult dogs.

Procedure—Single urine sample GAG concentrations and GCRs were measured in samples collected from 14 healthy dogs at the start of the 24-hour collection period. Twenty-four–hour total urine GAG excretions were determined from urine collected during a 24-hour period in the same 14 dogs. Total sulfated GAG concentrations were also measured in urine from these dogs after the urine had been stored at 4°C and −20°C for 1, 7, and 30 days.

Results—Urine GAG concentrations were not significantly different from baseline values after urine was stored at 4°C for up to 1 day and −20°C for up to 30 days. Neither single urine sample GAG concentration (IF, 0.422) nor GCR (IF, 0.084) was an adequate predictor of 24-hour total urine GAG excretion.

Conclusions and Clinical Relevance—Results of this study provide data that can be used to establish a reference range for 24-hour total urine GAG excretion in dogs and adequate conditions for sample storage. Contrary to findings in humans, there was no significant linear correlation between 24-hour total urine GAG excretion and single urine sample GCR in dogs, limiting clinical use of the single urine sample test. (Am J Vet Res 2006;67:51–55)

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Heparan sulfate, dermatan sulfate, keratan sulfate, and chondroitin sulfate are types of sulfated GAG chains found in human urine. Measurement of urinary excretion of GAGs in human medicine has been used to evaluate the pathogenesis of various diseases and treatments, including mucopolysaccharidosis, rheumatoid arthritis, urolithiasis, neoplasia of the urinary tract, extracorporeal shock wave lithotripsy, diabetes mellitus, interstitial cystitis, renal failure, glomerulonephritis, and renal amyloidosis. Of particular interest are altered GAG excretion in human patients with glomerular diseases. There is a paucity of reports on urine GAG measurements in dogs.

The urine GCR for single urine samples differed significantly between clinically normal humans and patients with primary glomerulonephritis or 2 forms of renal amyloidosis. In those studies, urine GCRs in humans with glomerulonephritis and amyloidosis were significantly decreased, compared with values for clinically normal humans. Analysis of these results suggests that the 1,9-DMB spectrophotometric assay of urine sulfated GAGs has the ability to distinguish clinically normal humans from those with primary glomerulonephritis and amyloidosis. The authors of those studies believed this test may also be able to differentiate between patients with glomerulonephritis and those with amyloidosis. In contrast, another study conducted by other investigators found an increase in urine GCR in humans with glomerulonephritis. Differences in GCR in these studies appear to be attributable to variation in preparation of buffer solutions, differences in the absorption wavelengths, questionable statistical analysis, and possible interfering substances.

The purpose of the study reported here was to determine whether the DMB spectrophotometric method could be used to accurately quantify concentrations of sulfated GAGs in urine samples obtained from dogs. Additionally, correlation of the single urine sample GCR with 24-hour total urine GAG excretion and methods of sample preservation were determined for urine samples obtained from clinically normal dogs.

Materials and Methods

Animals—Fourteen random-source adult dogs were used in the study. Dogs were considered healthy on the basis of results of physical examination, a CBC, serum biochemical analysis, ELISA to detect infection with Dirofilaria immitis, urinalysis, urine protein-to-urine creatinine ratio, and aerobic bacteriologic culture of urine samples. Dogs were fed a commercially available food formulated for dogs until 12 hours before the start of collection of urine samples. Dogs were allowed ad libitum access to water until the start of the collection of urine samples. The procedures in this study were approved by the Virginia Tech Animal Care Committee.

Procedure—Urine samples were collected from each dog for a period of 24 hours to determine the normal 24-hour total urine GAG excretion. Dogs were administered acepro-
mazine maleate (0.01 to 0.02 mg/kg, IM). Approximately 15 to 20 minutes later, each dog was weighed and then administered water (volume equivalent to 5% of body weight) via an orogastric tube to approximate daily maintenance water requirements and ensure hydration during the collection period. Neither food nor water was provided during the 24-hour collection period to prevent contamination of urine in the collection pans.

A urethral catheter was inserted into the urinary bladder of each dog, and the bladder was emptied at the beginning of the 24-hour collection period. Single urine sample creatinine and GAG concentrations were measured in this initial urine sample; analysis was conducted within 4 hours after collection of the initial urine sample. At the start of the 24-hour collection period, 3 mL of blood was collected from each dog and used to determine baseline (time 0) plasma concentrations of glucose, urea nitrogen, creatinine, phosphorus, calcium, total protein, albumin, globulin, total bilirubin, cholesterol, sodium, potassium, chloride, and total carbon dioxide and activities of alanine transaminase and alkaline phosphatase. Dogs were then placed in stainless-steel cages (1 × 1 m) equipped with underlying collection pans, attached drainage tubing, and collection bottles. Dogs were housed in the cages for 24 hours. Collection bottles were emptied every 2 hours. Urine for each dog was maintained in a separate container and stored at 4°C. At the end of the 24-hour period, all dogs were weighed and then administered acepromazine (0.01 to 0.02 mg/kg, IV) to rapidly tranquilize the dogs, which enabled us to catheterize each dog and avoid loss of urine from dogs urinating outside of the stainless-steel collection cages. After a urethral catheter was inserted into the urinary bladder of each dog, any remaining urine was removed from the bladder and added to the storage container for that dog. Total 24-hour urine volume was measured for each dog.

Urine creatinine concentration and urine GAG concentration were measured on an appropriately mixed aliquot of urine; measurements were conducted within 4 hours after the end of the 24-hour collection period. The 24-hour total urine GAG excretion was calculated by multiplying the 24-hour urine volume by the urine GAG concentration and dividing the product by body weight (in kilograms). Three milliliters of blood was collected at the end of the 24-hour collection period and used to determine the plasma creatinine concentration.

Aliquots (5 to 20 mL) of the appropriately mixed 24-hour total urine volume for each dog were placed into polyethylene vials and stored at 4°C and –20°C for 30 days. On days 1, 7, and 30 (day 0 was the first day of storage), urine samples were warmed to 23°C by use of a water bath. Aliquots were then mixed for 1 minute by use of an automated shaker. The GAG concentration of each aliquot was then measured.

**Assay of GAG concentration**—The procedure used to determine sulfated GAG concentrations in canine urine was adapted from a technique used in humans. This method was chosen because it is not affected by proteinuria, a problem detected with other versions of the DMB-based GAG assay. Chondroitin-4-sulfate was used as a representative sulfated GAG to approximate total sulfated GAGs. Briefly, a stock solution (10.0 mg/dL) of C-4-SO4 in deionized water was prepared. The same stock solution was used for all tests. Aliquots of stock solution were placed in separate test tubes and diluted appropriately with deionized water to achieve concentrations of 0, 0.25, 0.5, 0.75, 1.0, 1.5, 2.0, 3.0, 4.0, 5.0, 7.5, and 10.0 mg/dL for use in establishing an initial standard curve.

A stock solution of DMB was prepared by adding 11 mg of DMB to 1 L of 0.05M sodium acetate buffer (pH, 4.75). This stock DMB solution was stored at 23°C in a light-protected brown glass bottle with an airtight cap. Additional stock solution of DMB was prepared as needed and was stored for no longer than 1 month. No precipitate was observed at any time.

All urine samples were processed by centrifugation at 400 × g for 5 minutes. Supernatant was harvested and used for analysis. An aliquot (250 µL) of supernatant was added to 2.5 mL of DMB solution in each cuvette, the cuvette was inverted twice, and change in absorbance was measured within 1 minute. Change in absorbance caused by each urine sample was measured in triplicate at 520 nm, which was one of the absorbance peaks of the DMB–C-4-SO4 complex.

A spectrophotometer was used for determination of change in absorbance of C-4-SO4 stock solutions and urine samples. Disposable cuvettes with a 1-cm length of light path and volume of 4.5 mL were used. Change in absorbance of 2.5 mL of the stock solution of DMB (with deionized water used as a control sample) was determined in triplicate at 350, 400, 450, 500, 520, 535, 550, 600, 650, and 700 nm each day to ensure stability of the dye solution.

Each day that urine was assayed, a standard curve (C-4-SO4 concentrations of 1, 2, 4, 7.5, and 10 mg/dL) was created, and simple linear regression analysis was performed to determine the relationship between change in absorption and C-4-SO4 concentration. To determine whether there was a change in absorbance over time, C-4-SO4 concentrations of 0, 0.25, 1, 4, and 10 mg/dL were measured in triplicate at 1, 2, 3, 4, 5, 10, 20, and 30 minutes. No obvious change in absorbance was detected.

Regression lines for the sulfated GAG standard curves were assessed for linearity, and an R² value was calculated by use of commercially available software. An R² > 0.98 was considered strong evidence of a linear relationship, and the corresponding regression equations were used to calculate sulfated GAG concentration from the change in absorbance of each urine sample.

**Measurement of creatinine concentration**—Urine samples were centrifuged at 400 × g for 5 minutes. Supernatant was harvested and used for measurement of the creatinine concentration. Creatinine concentration was determined by an automated chemistry analyzer by use of the modified Jaffe procedure.

Endogenous Ccreat was calculated by use of the following equation:

\[
C_{\text{creat}} = \frac{(U_{\text{creat}} \times V)}{(P \times T \times X \times BW)}
\]

where \(U_{\text{creat}}\) is the urine creatinine concentration, \(V\) is the volume of urine, \(P\) is the mean plasma creatinine concentration in samples obtained at baseline and at the end of the urine collection period, \(T\) is the time of the collection period, and \(BW\) is body weight.

**Statistical analysis**—Data from all dogs that completed the 24-hour urine collection were used to study effects of urine storage on GAG concentrations. Only data from dogs with a \(C_{\text{creat}} \geq 2\) mLMin/kg were used for determination of single urine sample GAG concentrations and GCR. 24-hour total urine GAG excretion, and correlations among these variables.

The GAG concentrations of the stored urine samples were analyzed by use of a complete randomized block design to detect effects of time and temperature. Difference between the GAG concentration at time 0 and at each time and temperature combination was the variable used for analysis. A repeated-measures ANOVA was performed by use of commercially available statistical software. Values of \(P < 0.05\) were considered to indicate significant differences. Simple linear regression was used to determine the line of best fit for the relationship of 24-hour total urine GAG excretion with single urine sample
GCR and GAG concentration. The $R^2$ values were calculated by use of commercially available software as an estimate of the relationship among these variables.

**Results**

All 14 dogs completed the 24-hour urine collection period. The urine GAG concentration did not differ significantly from baseline values when samples were stored at 4°C or –20°C for 1 day or at –20°C for 30 days, but concentrations were significantly different at all other time and temperature combinations (Table 1). Urine GAG concentrations changed in a similar manner for both storage temperatures.

Of the 14 dogs that completed the 24-hour urine collection, 10 had a $C_{\text{creat}} > 2$ mL/min/kg (mean ± SD, 2.581 ± 0.352 mL/kg/min). This group consisted of 6 spayed females, 3 sexually intact males, and 1 neutered male. Mean body weight was 21.57 kg. The age of each dog was unknown, but all were adults. Data from the 4 dogs with a $C_{\text{creat}} < 2$ mL/min/kg were excluded from determination of values for clinically normal dogs.

For the 10 dogs with a $C_{\text{creat}} > 2$ mL/min/kg, mean ± SD 24-hour total urine GAG excretion was 1.586 ± 0.461 mg/kg/d. Mean GCR for the 24-hour total urine sample was 0.044 ± 0.012. Mean single urine sample GAG concentration and GCR were 5.007 ± 1.588 mg/dL and 0.023 ± 0.01, respectively. No significant linear relationship could be detected between single urine sample GAG concentration and 24-hour total urine GAG excretion or single urine sample GCR and 24-hour total urine GAG excretion (Figures 1 and 2).

Therefore, neither single urine sample GAG concentration ($R^2$, 0.422) nor GCR ($R^2$, 0.084) was an adequate predictor of 24-hour total urine GAG excretion. The GCR of the 24-hour total urine sample was consistently greater than the single urine sample GCR (Figure 3).

**Discussion**

Urine GAG concentrations remained stable when urine samples were stored at 4°C or –20°C for 1 day.
and −20°C for 30 days, but concentrations were significantly different at all other time and temperature combinations. Bacterial growth was considered as a potential source of GAG production; however, this appears unlikely on the basis that these dogs had normal results for urinalyses, bacterial urinary tract infection does not affect GAG concentrations in humans, and bacteria would not be expected to proliferate at 4°C.17 To our knowledge, the effect of storage times and temperatures on urine GAG concentration has not been reported elsewhere.

The standard curve for sulfated GAGs was linear for the range of 0 to 7.5 mg/dL, which is consistent with results for humans.2 The mean single urine sample GAG concentration and GCR in the 10 dogs completing the 24-hour urine collections were of similar magnitude to values reported6,7,15,17 for humans determined by use of various methods of GAG measurement. Analysis of these findings supports the contention that the assay accurately measured sulfated urine GAG concentrations in clinically normal dogs.

Perhaps the most important finding in the study reported here was that for the conditions of the experiment, single urine sample GCR does not correlate well with 24-hour total urine GAG excretion. This correlation is important because if single urine sample GCR estimates the 24-hour total urine GAG excretion, it then could be used as a convenient measurement requiring only a single sample for comparison of GAG excretion among animals. If single urine sample GCR cannot be used, a 24-hour urine collection would be necessary, which is impractical for routine use in veterinary medicine. Additionally, this would require an indwelling catheter, and the effects of an indwelling catheter on GAG concentration are unknown.

Several possible reasons exist for lack of a correlation between single urine sample GCR and 24-hour total urine GAG excretion. Physical differences between the single urine samples and 24-hour urine samples may have resulted because urine for the latter was in contact with the stainless-steel cage and collection tubing. Effects of dander, hair, and interactions with the tubing on GAG and creatinine concentrations are unknown. Lack of correlation also could have resulted if excretion of GAGs or creatinine were not constant, especially if there is nonparallelism in their fluctuation throughout the day. Nearly all reports in which investigators have measured urine GAG concentrations have expressed the results as a ratio in relation to the urine creatinine concentration. However, there is conflicting information in humans as to whether GCR corrects the GAG concentration for variable states of dehydration or approximates 24-hour total urine GAG excretion2,17,19,20. In 1 study,17 investigators found that GAG excretion measured in single, random urine samples had good linear correlation with 24-hour total urine GAG excretion. In another study,17 investigators used the same GAG assay that we used in the study reported here and found daily and circadian urine excretions of GAG were constant in 5 humans. However, when urine GAG concentrations are estimated by measuring hexuronic acid or uronic acid concentrations, there may be nonparallelism in daily fluctuation of GAG and creatinine concentrations.19,20 The GCR measured in a single random sample may fluctuate throughout the day, and there may also be day-to-day fluctuations, but the GCR for 24-hour urine samples remains consistent from day to day.19 In the study reported here, the single urine sample GCR was measured in a sample collected from each dog in the morning and was lower than the GCR for the total 24-hour urine sample in all 10 dogs (Figure 3). This implies that excretion of GAGs or creatinine is not constant in dogs. Differential effects of hydration status on production, excretion, and reabsorption of creatinine and GAGs should be considered, but have not been reported in humans or dogs to our knowledge.

Water gavage was used in place of ad libitum consumption of water to ensure hydration and prevent contamination of the collected urine. However, this gavage did add the variable of forced diuresis and may have altered GAG excretion during the subsequent collection period. Additional studies involving ad libitum access to water and collection of single, random urine samples multiple times throughout a 24-hour period may help clarify any contribution hydration status and time of sample collection may have had on our findings. Such studies may also reveal conditions for which the single urine sample GCR is a viable measurement for clinical studies.

The study reported here provided data that can be used to establish a reference range for 24-hour total urine GAG excretion in dogs. At this point, it must be concluded that single urine sample GCR is not an adequate predictor of 24-hour total urine GAG excretion. On the basis of analysis of results of our study, use of 24-hour urine collections would be needed to make comparisons among specific animals. Additionally, this information should cause clinicians and researchers to use caution when interpreting results of experiments in which single urine sample GCR is used to compare GAG excretion in humans and in cats with interstitial cystitis.

References


